REMARKS

Claims 27-36, 38-47, 49-59 and 61-68 are pending, applicants having cancelled claims 37, 48 and 60 in a prior amendment.

New *dependent* claims 70-73, fully supported by the originally filed specification, have been added.

Applicants herein Request Continued Examination (RCE).

Applicants thank the Examiner for withdrawing the prior new matter rejection, and for withdrawal of the rejection of kit claims under 35 U.S.C. § 103(a),

The maintained rejection of claims 27-34, 36, 38-45, 47, 49-57, 59 and 61-68 under 35 U.S.C. § 103(a) as being unpatentable over Herman et al. (U.S. Patent 6,017,704, Jan. 25, 2000) as applied in view of Wittwer et al (U.S. Patent 6,140,054, Oct. 2000) is acknowledged. Applicants have respectfully traversed this rejection, but have nonetheless amended the independent claims to further clarify the distinguishing real-time aspect subject matter.

The maintained rejection of claims 27-35, 38-46, 47, 49-58 and 61-67 under 35 U.S.C. § 103(a) as being unpatentable over Herman et al. (U.S. Patent 6,017,704, Jan. 25, 2000) as applied in view of Witcombe et al (U.S. Patent 6,270,967, Aug. 2001) is acknowledged. Applicants have respectfully traversed this rejection.

The Examiner's maintained rejection under obviousness-type double patenting are acknowledged, and applicants reaffirm their intention to timely file a Terminal Disclaimer upon indication of allowable subject matter.

No new matter has been added.

35 U.S.C. § 103 Rejections

Herman in view of Wittwer:

The Examiner has maintained the rejection of claims 27-34, 36, 38-45, 47, 49-57, 59 and 61-68 under 35 U.S.C. § 103(a) as being unpatentable over Herman et al. (U.S. Patent 6,017,704, Jan. 25, 2000) as applied in view of Wittwer et al (U.S. Patent 6,140,054, Oct. 2000) (Office

Action of 30 June 2005, section 6, pages 3-9).

Specifically, the Examiner alleges that Herman teaches: "contacting the sample of genomic DNA with bisulfite"; "amplifying the converted nucleic acid with primers that distinguish between methylated and unmethylated DNA such that at least one oligonuclotide probe is a CpG specific probe [actually Herman teaches ASO probes]; and "detecting the methylated nucleic acid based on amplification mediated change ...." The Examiner additionally states that Herman teaches that amplified products are preferably identified by sequencing, and further asserts that allele-specific oligonucleotide (ASO) probes are equivalent to CpG specific probes, and that detection of such probes is among Herman et al's listed means for sequencing the amplified products. The Examiner appreciates that the detection of Herman et al is and 'end-analysis' approach; that is, ***subsequent*** to amplification (unlike the present real-time methods), but the Examiner alleges that while Herman does not teach using FRET probes to detect allele specific differences, Wittwer et al nonetheless teach identification of polymorphisms using FRET probe pairs in combination with PCR and standard melting curve analysis (*Id*, at lines19-20).

The Examiner states that it would have been *prima facie* obvious to one of ordinary skill at the time of the present invention to have modified and improved the method of Herman by using ASO probes with the allele specific detection method of Wittwer (premised on an analogy between genomic alleles and bisulfite-treated DNA).

Applicants respectfully traverse this rejection based on the arguments of record and as further clarified and extended herein.

First, neither Herman nor Wittwer teach or suggest detection during amplification that is based on amplification-mediated probe displacement.

Second, neither Herman nor Wittwer teach or suggest real-time detection in the present sense, but rather Wittwer teaches a limited *quasi*-real-time method requiring melting curve determination either after the PCR amplification is completely finished, or after each extension cycle during the amplification, and even here the methods are not based on amplification-mediated probe displacement.

With respect to the ***first*** point, detection in Wittwer is not based on probe displacement. Wittwer rather uses adjacently hybridizing pairs of oligonucleotides (one of which is designed to be genotype-specific) wherein each member of the pair has one fluorophore of a fluorescence energy transfer pair, so that upon hydribization (not probe displacement) of the pair to their complementary sequences resonant energy is detectably transferred from the donor to the acceptor fluorophore. Where the fluorophore pair is not in fluorescence energy transfer relationship (i.e., when they are not hybridized to their adjacent complementary sequences) the donor fluorophore will not produce an increased fluorescence by the acceptor. The consequence of this is that meaningful detection in Wittwer can only effectively occur either at the end of the amplification reaction or, in particular embodiments, between extension cycles during the melting curve analysis (see following discussion of quasi-real-time).

With respect to the ***second*** point, despite the Wittwer references to "real-time" and "continuous," the Wittwer method is not a real-time method in the instant sense, but is either an 'end-point' method monitoring fluorescence as a function of temperature to determine a "PCR product melting curve" at the end of the completed amplification reaction (using "an appended analytical cycle *after* amplification"), or at best a ***quasi*** real-time method based on monitoring fluorescence and fluorescence as a function of temperature to determine a "PCR product melting curve" at the end of individual extension cycles of the amplification reaction.. In either case, the "generated melting curve is then compared to the known melting curve for the mutant, normal or polymorphic sequence to determine the sequence of the target nucleic acid" (column 4, lines 14-17). Significantly, in the quasi-real time embodiments of the Wittwer method, the FRET probe pair signal is completely abolished every time the PCR temperature is raised (dissociating and thus separating the FRET pair), and a melting curve must be determined and compared. Therefore, the Wittwer methods are not in fact real-time in the presence sense, and rather measure a gradual *incremental*, non-continuous increase in the post-amplification melting curve assay, or in quasi-real-time embodiments the post-extension reaction melting curve assay with repeated PCR cycle Specifically, as discussed in detail in Wittwer:

"In accordance with one aspect of the present invention, real time fluorescence monitoring of the PCR reaction is used to acquire product melting curves during the PCR reaction. The temperature cycles of PCR that drive amplification alternately denature the accumulating product and the fluorescently labeled hybridization probes at a high temperature, and anneal the primers and the hybridization probes to the product at a lower temperature. The transition temperatures of the fluorescently labeled hybridization probes depend primarily on GC content and length. If a probe is designed to hybridize internally to the PCR product, the melting temperature of the probe also depends on GC content, length, and degree of complementarity to the target. Plotting fluorescence as a function of temperature as the sample is heated through the dissociation temperature of the product gives a PCR product melting curve. The shape and position of this DNA melting curve is a function of GC/AT ratio, length, and sequence, and can be used to differentiate amplification products separated by less than 2.degree. C. in melting temperature. Thus continuous monitoring of fluorescence during the PCR reaction provides a system for detecting sequence alterations internal to the PCR primers by resonance energy transfer and probe melting curves."

Wittwer at column 15, line 55 through column 16, line 10 (emphasis added). Additionally, as discussed under representative Example 8 discussing genotyping of C282Y and H63D sites with adjacent fluorescent hybridization probes:

"Forty repeats of a 2-temperature cycle were performed (94.degree. C. for 0 s and 62.degree. C. for 20 s with programmed transitions of 20.degree. C./s). Fluorescence was acquired once each cycle for 50 msec per sample at the end of the combined annealing/extension step. An appended analytical cycle after amplification allowed immediate genotyping by derivative melting curves."

Wittwer at column 24, ll. 53-58 (emphasis added). Accordingly, meaningful detection in Wittwer effectively occurs either at the end of the complete amplification reaction or, in so-called 'real-time' embodiments, between extension cycles, during the melting curve analysis. Therefore, references in Wittwer to 'real-time' and 'continuous monitoring' are in actuality at best quasi-real-time in nature, and are fundamentally distinguishable from the instant methods that are truly real-time, where detection is based on amplification (extension) mediated probe displacement. Thus, Herman and Wittwer teach away from the present invention, which is real-time and comprises detection based on amplification-mediated probe displacement.

Applicants have nonetheless amended independent claims 27, 38 and 50 to recite

"detecting in real-time during the amplification, the methylated nucleic acid based on amplification-mediated probe displacement." to further clarify the *real-time* aspect of the presently claimed subject matter. Conforming amendments have been made to independent claim 61.

Support for the recitation of "detecting in real-time during the amplification" is found throughout the originally-filed application, which is replete with references to "real-time" PCR and also for example at page 12, lines 30 and 31, teaching contemporaneous (*i.e.*, real-time) detection.

New *dependent* claims 70-73, reciting "wherein detecting based on amplification-mediated probe displacement, comprises detecting amplification-mediated change of probe fluorescence" have been added. Support for these claims is found throughout the specification, for example at page 6, lines 25-27 and page 15, lines 21-22. No new matter has been added.

Applicants, therefore, respectfully request withdrawal of the Examiner's rejection of claims 27-34, 36, 38-45, 47, 49-57, 59 and 61-68 under 35 U.S.C. § 103(a) in view of applicants' amended claims 27, 38, 50 and 61.

Herman in view of Witcombe:

The Examiner has maintained the rejection of claims 27-35, 38-46, 47, 49-58 and 61-67 under 35 U.S.C. § 103(a) as being unpatentable over Herman et al. (U.S. Patent 6,017,704, Jan. 25, 2000) as applied in view of Witcombe et al (U.S. Patent 6,270,967, Aug. 2001).

Specifically, the Examiner alleges that while Herman does not teach using TaqMan probes to detect allele specific differences, Witcombe et al nonetheless do (Office Action of 30 June 2005 at page 11, paragraph 8).

Applicants respectfully traverse this rejection, based on the fact that neither Herman nor Witcombe teach or suggest use of CpG-specific probes for real-time detection of DNA methylation. In fact, as described below, both Herman and Witcombe teach away from such an application by specifying that methylation detection is based on of CpG-specific primers, and not probes.

First, as is clear in the prosecution record, Herman teaches methyation assays based on biased primers (*e.g.*, methylations-specific primers), and while Herman may teach 'end-point' sequencing of amplification reactions using ASO probes, Herman does not teach real-time detection using any probe (ASO, CpG-specific or otherwise) , and teaches amplification by CpG-specific primers.

Second, and contrary to the Examiner's assertion (in reference to Figure 10, col. 8, lines, 58-62), Whitcombe does not use TaqMan (real-time probes) that are specific for allele discrimination of the ASO element. Rather, for allele discrimination of the ASO element, Whitcombe uses "tailed diagnostic primers" having a "tag region" and a "detector region" on the non-hybridizing tail. Therefore, direct discrimination of the ASO element is by hybridization of the non-tail diagnostic portion of the primer to the genomic ASO sequence, and the detector region of the non-hybridizing tail does not hybridize to or discriminate the ASO element (see col. 1, lines 35-67 and extending to col. 2. lines 1-25). Specifically (and with reference to Figure 10, col. 8, lines 58-62 cited by the Examiner), Whitcombe teaches a two-phase assay, wherein in the *first* phase an extension product is initially generated by extending the "tailed diagnostic primer" (present in relatively low amounts to avoid mis-priming errors) followed by production of a "further primer extension product" that is reverse-primed off the initial extension product using a "further primer." The "further primer extension product" includes (at the 3'-end thereof) a sequence complementary to the 'tag' and 'detector region' of the tail of the tailed diagnostic primer (*Id*). In the *second* phase a tag primer (in relatively high concentrations relative to the initial diagnostic primer) is then used to drive many PCR cycles in the presence of (in particular real-time embodiments), *e.g.*, a TaqMan probe that is complementary to the 'detector region' sequence. Significantly, the detector region sequence is not designed to discriminate the ASO element/allel (*Id*), and thus the various "detection probes" (*e.g.*, TaqMan probes) that are discussed that recognize the detector region sequence are not ASO specific. Thus, while TaqMan probes are used in the context of allele discrimination of an ASO element, such use is *indirect*, and no where does Whitcombe teach or suggest using the TaqMan or any other real-time probe that is

CpG-specific, or even ASO-specific. The "detection probes" of Whitcomb are not ASO-specific at all, and rather non-discriminately hybridize to the "detector region" on the non-hybridizing tail of the detection primer.

Therefore, while Whitcombe may teach a real-time assay, Whitcombe does not teach detection based on amplification-mediated displacement of a CpG-specific probe. Rather, in particular embodiments, Whitcombe ***teaches away*** from the present invention by teaching detection based on amplification-mediated displacement of a **non**-CpG-specific probe. Moreover, Whitcombe's two-phase assay design is explicitly designed to minimize 'mispriming' and 'primer-dimer' effects:

> "The present invention comprises a two stage procedure wherein as a first stage the initial interaction between a diagnostic primer comprising tag and detector regions and a sample template may conducted at optimum hybridisation stringency. Any primer extension products are then amplified using a further primer. As a second stage the above extension products are then amplified using a tag primer and the further primer. Accordingly, whilst mispriming may still initially occur the overall level may be significantly reduced."
>
> "We now disclose the use of diagnostic and further primers which are genome specific at their 3'-termini but which carry a detector region and common extensions (tags) at their 5'-termini. These are used in combination with a common tag primer which can prime from the complement of the tag sequence in extension products of further primer(s). Thus whilst primer dimers and other inter-primer artefacts could occur during first phase diagnostic priming, these cannot be amplified during subsequent rounds of tag specific priming. It will be appreciated that the diagnostic primers are conveniently used at concentrations which allow satisfactory priming on their genomic template(s) but do not allow significant PCR amplification."

Whitcombe at column 3, ll. 8-65 (emphasis added).

Therefore, to mimimize mispriming and primer-duplex effects, person of ordinary skill in the art would not have been motivated toward the instant invention, and there is no teaching or suggestion to combine real-time allele discrimination methods of Whitcombe with an end-point methylation assay such as that of Herman to arrive at applicant's invention, which comprises real-time detection based on based on amplification-mediated displacement of a CpG-specific probe. In fact, Whitcombe teaches away from the instant invention by teaching ASO-specific primers, and also by teaching a two-phase assay design.

Applicants, therefore, respectfully request withdrawal of the Examiner's rejection of claims 27-35, 38-46, 47, 49-58 and 61-67 under 35 U.S.C. § 103(a), in view of Herman et al, and further in view of Whitcombe.

Secondary Indicia of Objective Non-obviousness

While applicants respectfully contend that the claims as currently amended are allowable based on the arguments outlined above, applicants wish to further comment on the *secondary indicia of objective non-obviousness* discussed in applicants' last Response. The Examiner, in response to applicants' discussion of the age of the references discussed in relation to applicants' priority date, stated that "contentions that the references are old are not impressive absent a showing that the art tried and failed to solve the same problem nontwithstanding its presumed knowledge of the references. Applicants want to point out that the Whitcombe patent asserted by the Examiner was based on a PCT application that was published in 1997 (PCT/GB97/01163), and was therefore known in the art. As previously stated, *real-time* assays were in the art as early as 1995 and 1996, and MSP technology was published in 1996. Therefore, more than four years transpired after the advent of real-time technology, more than three years transpired after the advent of MSP, and more than two years transpired after the publication of PCT/GB97/01163 before the present applicants were the first to teach combining bisulfite treatment of DNA, methylation-specific probes and *real-time* PCR to address a long-standing need for providing efficient and quantitative real-time methylation assays, based on methylation-sensitive probes, the detection of which is based on amplification-mediated probe displacement. In fact, during this period and contrary to the Examiner's assertion that it would have been *prima facie* obvious to one of ordinary skill at the time of the present invention to have modified and improved the method of Herman by using ASO probes with the allele specific detection method of Whittcombe the *skilled* people in the art were actually *teaching away* and also failing to solve the problem solved by the present invention by introducing and promoting *end-point* fluorescence assays and *fluorescent melting curves* after multiple PCR cycles, along with other solutions (not cited by the Examiner)

involving the use of methylation-sensitive restriction enzymes, and primer-extension of nonmethylation-sensitive primers. Therefore, applicants content for the record that there is a "showing that the art tried and failed to solve the same problem nontwithstanding its presumed knowledge of the references."

Obviousness-type Double Patenting Rejection

The Examiner has maintained the rejection of claims 27-32, 38-43, 50-55 and 61-67 under the judicially created doctrine of obviousness-type double patenting as being unpatentable in view of claims 1-26 of U.S. Patent No. 6,331,393 (December 18, 2001) (Office Action of 28 October 2004, at page 18).

Applicants maintain their intention to timely file a Terminal Disclaimer upon notification of allowable subject matter.

Applicants respectfully request entry of the present Response and Amendment, and reconsideration and allowance of all pending claims 27-36, 38-47, 49-59 and 61-68, and of new claims 70-73 of the present application.

Respectfully submitted,

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